Alterations of the Retinoblastoma and P16 Pathway Correlate with Promoter Methylation in Malignant Fibrous Histiocytomas

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Abstract. *Recent reports indicate that the alterations in the p16 and pRb pathways can influence tumour progression and poor prognosis in several tumours. The objective of this study was to analyse p16 and pRb expression in161 patients with malignant fibrous histiocytomas (MFH). By immunohistochemistry, p16 and pRb were demonstrated in 25% and 56% of MFH, respectively. Cox regression analysis demonstrated an independent prognostic influence of both genes. Generally, the loss of p16 and pRb expression correlated with poorer prognosis. Promoter methylation of p16 was found in 16/42 of p16 negative MFH and of pRb in 2/42 of pRb-negative MFH. It can be concluded that p16 and pRb alterations play an important role in the progression of soft tissue sarcomas.*

Malignant fibrous histiocytoma (MFH) belongs to the group of malignant soft tissue tumours and represents the most common malignant soft tissue tumour of the late adulthood (1-3). Not only the subtype classification, but other histopathological features and factors can be helpful in establishing the prognosis and therapeutic strategy for each individual tumour. Recent tumour biological investigations have shown that tumour development can be caused by a series of genetic alterations which specifically comprise changes in ploidy status, proliferation and tumour suppressor gene defects, *e.g.,* the mutated p53 or retinoblastoma gene (4-6).

The retinoblastoma gene intervenes in cell cycle regulation and encodes a 105 kDa phosphoprotein, which accumulates in the cell nucleus. Both the cell content of retinoblastoma gene protein as well as phosphorylation of the protein are cell-cycle dependent. It is assumed that the under-phosphorylated form of pRb involves the functionally active, proliferation-inhibiting protein in the G0-middle G1 phase (7-9).

Another cell cycle regulator is p16. P16 is also called INK4/MTS1 and acts as a tumour suppressor gene. The negative influence of p16 on proliferation has been reported. P16 is known to indirectly inhibit the phosphorylation of pRb (10, 11).

The main objective of this study was to examine the prognostic relevance of the expression of cell cycle regulators (pRb, p16) in malignant fibrous histiocytomas. In order to elucidate the reasons for a lack of p16 (pRb) protein expression in samples studied, an analysis of promoter methylation for both genes was performed.

Materials and methods

Patient characteristics. A well-characterised cohort of 161 primary MFHs, corresponding to patients treated at the teaching hospitals of the University of Göttingen and the University of Krakow between 1992 and 2001, was the focus of this study. There were no differences between patients treated in Poland (n=116) and Germany (n=45) with respect to disease-related survival (log-rank test). The age of the patients ranged between 4 and 90 years (mean 61 years); 45% of the MFH patients were females and 55% were males.

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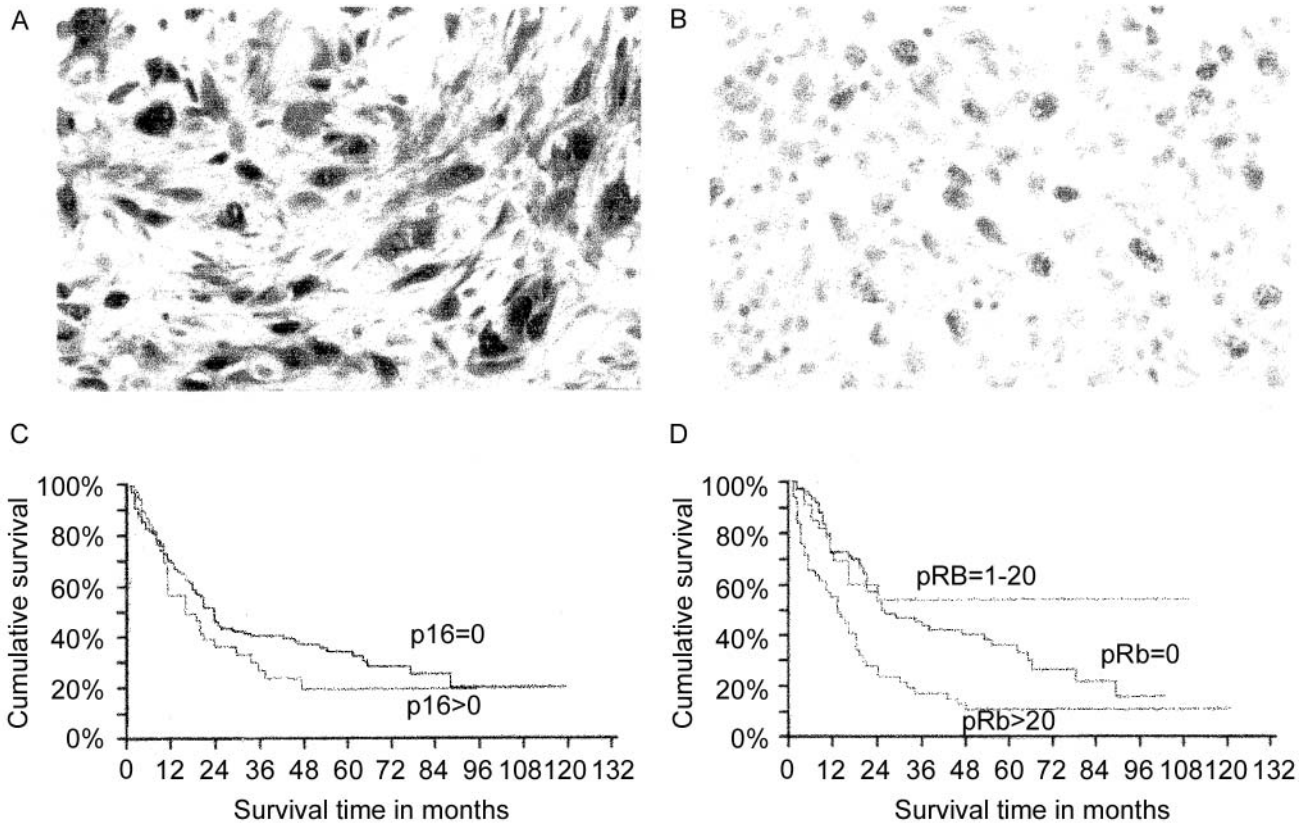

Figure 1. *A) P16-positive cells in malignant fibrous histiocytomas. B) Retinoblastoma positivity in malignant fibrous histiocytomas. C) Kaplan-Meier survival curves for p16-positive and -negative malignant fibrous histiocytoma (MFH) patients. D) Kaplan-Meier survival curves demonstrating the relationship between pRb expression and survival time of MFH patients. Note that patients were divided in 3 groups: i) cases without pRb expression; ii) cases with low expression (1-20%); and iii) cases with high expression (>20%).*

The anatomic localisation of these tumours was distributed as follows: head and neck (n=27), upper extremities (n=19), lower extremities (n=86) and trunk (n=29). Thirty-six tumours (22%) showed superficial localisation, while 117 (73%) had deep localisation. Tumour depth could not be determined in 8 cases. The size of the tumours ranged between 0.6 and 36 cm (median=8 cm). Thirty-nine (24%) of the neoplasms were assigned to pathological stage pT1 and 122 (76%) tumours to pT2. In 43 patients (27%), distant metastases were clinically present at the time of the primary diagnosis. In 2 patients, locoregional lymph node metastases were assessed by histopathology. All patients were observed from the time of diagnosis up to the end of the study period (October 2001), and the disease-related survival within this period was documented. Non-MFH-related fatalities were excluded from the investigation. The ethical approach of this study was aproved by the Ethical Center of the Adam Mickiewicz University in Poznan, Poland.

Immunohistochemistry. The tumour tissue was routinely fixed in 3.6% formaldehyde and embedded in paraplast. Three-μm thick serial sections were prepared by the same technician to ensure that the quality of the sections remained consistent. The histological sections were mounted on uncoated slides and were deparaffinated by xylol and then transferred to a descending alcohol series and rinsed with distilled water. The following antibodies were utilised for this study: mouse monoclonal antibody against human p16, clone G175-405 and mouse monoclonal antibody against retinoblastoma protein (pRb) clone G3-245 (both antibodies from Pharmingen, San Diego, USA).

The immunohistochemical reactions in the paraffin-embedded tumour tissue were carried out using the Stravigen Multilink kit (Biogenex Laboratories, Hamburg, Germany). Before incubation with primary antibodies, the sections were heated for 10 min on a heat plate (85˚C) in citrate buffer (pH=6). Afterwards, incubation with the primary antibodies was carried on overnight at 4˚C at an antibody concentration of 1:20 for retinoblastoma protein and 1:50 for p16. The histological specimens were then rinsed with Tris buffer solution and incubated at room temperature with link (Stravigen Multilink) for 45 min. After the detection reaction was performed using a label (Stravigen Multilink) in combination with chromogen fast red (Biogenex Laboratories), the nuclei were counterstained with haematoxylin. In control reactions, primary antibodies were omitted. The sections were evaluated by CAS200 image analysis system and the results were expressed as percentages of immunolabeled cells (indices).

Table I. *P16 and pRb indicates in MFH primary tumors in relation to histopathological grade of malignancy.*

Parameter	Grade	Minimum	25th Percentile	Median	75th Percentile	Maximum
p16 index	I	0	0	0	0	90
	II	0	0	0	30	96
	III	0	0	0	1	85
pRb index	I	0	0	1	20	60
	II	0	0	0	8	95
	III	0	0	19	40	90

Analysis of p16 and pRb promoter methylation. The probes were excised from the paraffin blocks containing the tumour tissue for histopathological diagnosis. One μg of DNA isolated from MFH and non-neoplastic control tissue was treated with sodium bisulfite using the CpGenome DNA Modification Kit (Intergen Company, Oxford, UK) according to the manufacturer's instructions. Two μl of modified DNA (1/5 volume) were used for PCR amplification. The primers were: p16 sense 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3' and anti-sense 5'-GAC CCC GAA CCG CGA CCG TAA-3' for methylated sequences, p16 sense 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3' and anti-sense 5'-CAA CCC CAA ACC ACA ACC ATA A-3' for non-methylated sequences, pRb sense 5'-GGG AGT TTC GCG GAC GTG AC-3' and anti-sense 5'-ACG TCG AAA CAC GCC CCG-3' for methylated sequences, pRb sense 5'-GGG AGT TTT GTG GAT GTG AT-3' and anti-sense 5'-ACA TCA AAA CAC ACC CCA-3' for non-methylated sequences. PCR conditions were those described by Chim *et al.* (12). The PCR fragments were separated on a 3% agarose gel.

Statistics. The data were analysed using the statistical analysis system (SAS, Version 7.5) on an IBM-compatible PC under windows XP and had been previously scanned into the spread sheet (Microsoft Excel XP), where they were made available to the statistics program *via* an ODBC (open database connectivity).

The median and the other quantiles were calculated for all expression indices. The data were presented as box-plots. Mann-Whitney-*U*-tests were used for the paired group comparisons. The Kaplan-Meier method was employed to calculate the survival rates (13). Cox regression was the multivariate method used for the predicting the survival rate based on several parameters (14).

Results

Immunohistochemical results. The tumour suppressor gene protein p16 was localised in nuclei by immunohistochemistry (Figure 1A). P16 expression was found in 25% of the MFH cases. P16 expression was higher in grade II tumours than in grade I tumours. The p16 index in grade III tumours was as low as in grade I MFH (Table I).

The reaction product for the retinoblastoma gene protein was detected in the nuclei (Figure 1B). In the control reactions, the retinoblastoma gene protein antibody selectively labelled the epithelial cells of the epidermis with suprabasal localisation. Retinoblastoma gene expression was found in 56% of the sarcomas investigated. The pRb index varied between 0 and 95% (median 1). Grade II tumours showed the lowest pRb expression (median 0) and grade III the highest index (median 19) (Table I).

Relationship between p16 and pRb expression and survival rates. Cases of MFH with an expression of p16 (index>0) and a lack of p16 expression (index=0) did not differ significantly with regard to the probability of survival (Figure 1C). Cases of MFH with a high expression of the pRb (index>20) showed a lower probability of survival than cases with a median (index 1-20) or low expression (index=0) (Figure 1D).

Likewise, the combination of p16 and pRb showed that the highest 5-year-survival rates were observed in patients with zero values for both parameters. An exact χ^2-test showed that the difference in 5-year-survival rates were significant ($p<0.001$). Moreover, it was shown that patients for whom these parameters were normal (p16=0 and pRb=0) had the significantly highest 5-year survival rates of the sample ($p=0.004$), as verified by χ^2 analysis.

The results of the Cox regression for predicting survival based on categorised cell-cycle regulator (CCR) indices (p16, pRb), showed, in both forward and backward parameter selection models, an independent prognostic role of p16 and pRb in malignant fibrous histiocytomas (Table II).

Relationship between p16 and pRb expression and promoter methylation. Promoter methylation of p16 and pRb was analysed in 42 p16- and pRb-negative MFH. Sixteen out of 42 cases investigated contained hypermethylated p16 promoter sequences. In contrast, only 2 cases revealed pRb methylation (Figure 2).

Discussion

The present study investigated 161 cases provided by the Pathological Institutes at the University of Göttingen and the University of Krakow. Comparatively speaking, the processed tumour samples belong to one of the largest

Table II. *Results of the Cox regression for predicting survival based on categorized CCR indices (p16, pRb).*

Forward parameter selection				
Remaining independent prognostic parameters	*P*-value	Change of hazard rate per unit of parameter	Lower bound of 95% confidence	Upper bound of 95% confidence
p16 index	0.003	1.1%	0.4%	1.9%
pRb index	0.001	2.2%	1.3%	3.2%
Backward parameter selection				
Remaining independent prognostic parameters	*P*-value	Change of hazard rate per unit of parameter	Lower bound of 95% confidence	Upper bound of 95% confidence
p16 index	0.019	0.9%	0.2%	1.7%
pRb index	0.001	2.3%	1.4%	3.2%

CCR: cell-cycle regulator.

published MFH series that has been examined utilizing special techniques. The patients samples of other studies, *i.e.,* Weiss and Enzinger, Pezzi *et al.,* Hashimoto *et al.* and Doussal *et al.* were more extensive, but were only investigated with regard to the prognostic relevance of grading parameters (15-18).

In this study, the association between the altered expression of p16 and pRb and patients survival rate was demonstrated. Both markers demonstrated an independent influence on survival, as confirmed by Cox regression analysis.

It is well known that loss of expression of p16 and pRb is either connected with mutation or with the methylation of promoter region. Mutations that disable the retinoblastoma pathway are common in human cancer. Cells with an altered retinoblastoma gene pathway produce aberrant Mad2 expression and mitotic defects leading to aneuploidy (19). These mutations promote tumour development by deregulating the E2F family transcription factors leading to uncontrolled cell proliferation.

In this study, loss of p16 and pRb expression correlated with promoter methylation of these genes. This observation was especially true for p16. Defects in p16 and pRb are very often simultaneous as reported for several tumours (20) and also confirmed by this study. Our results demonstrate p16 promoter methylation in approximately 35% of p16-negative MFH and are in accordance with the results obtained on other sarcomas. The promoter methylation of p16 was reported for highly malignant chondrosarcomas (21). The methylation of the p14 and p16 promoter region was also reported in 1 out of 6 human osteosarcoma cell lines. In contrast, alterations of the retinoblastoma pathway were reported in up to 80% of osteosarcoma cases, suggesting that deregulating the G1 to S cell cycle checkpoint may be an almost constant change in the pathogenesis of osteosarcomas (22).

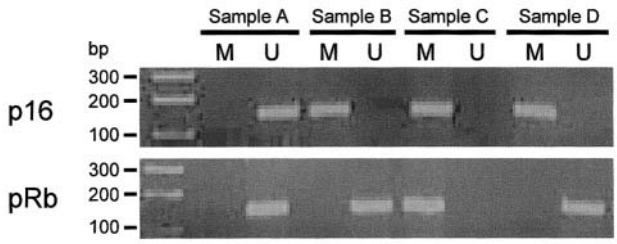

Figure 2. *Analysis of the p16/ pRb promoter methylation status in malignant fibrous histiocytoma samples. The results of four samples lacking expression of both, p16 and pRb are shown. The PCR fragments resulting from the DNA extracted and modified by bisulfite treatment were separated on 2% agarose gel. Data show that sarcoma samples B, C and D contain hypermethylated p16 promoter sequences. In contrast, only sample C reveals pRb methylation.*

In the group of chondrosarcomas all tumours that showed chromosome 9 alterations, also showed loss of heterozygosity and p16 expression. A lack of correlation between LOH, promoter methylation and protein expression indicated that a locus other than CDKN2a/p16 must be the target of LOH (23). A correlation between p16 protein expression and tumour grading indicated that loss of p16 expression may be an important event during tumour progression.

Inactivation of the p16 gene in leiomyosarcomas of soft tissue was correlated with decreased protein expression of p16, promoter methylation and poor prognosis. Eight out of 15 cases with decreased expression of p16 revealed methylation of promoter region (24).

P16 showed an aberrant promoter methylation in 12 out of 19 cases of primary angiosarcoma of the liver and influenced poor prognosis (25). The absence of p16

protein could have not only influence on prognosis but also on the result of therapy. The absence of the p16 protein led to higher glucocorticoid receptor transactivation activity and reduced cell sensitivity to dexamethasone (26).

In conclusion, the results of the present study demonstrate for the first time, an independent prognostic role of p16 and pRb in MFH. Methylation of promoter region of these genes was also demonstrated and was found, especially in the case of p16, to correlate with poor prognosis of patients with MFH.

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